Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 712–715, February 1983
Biochemistry

Sterol synergism in yeast

(cholesterol/ergosterol/plasma membrane/phospholipids)

MALATHI RAMGOPAL AND KONRAD BLOCH*

James Bryant Conant Laboratories, Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138

Contributed by Konrad Bloch, November 5, 1982

ABSTRACT **Sterol synergism as previously observed [Dahl, C. E., Dahl, J. S. & Bloch, K. (1980) *Biochemistry* 19, 1462–1467] and defined as a greater-than-additive growth response to pairs of sterols by *Mycoplasma capricolum* [Dahl, J. S., Dahl, C. E. & Bloch, K. (1981) *J. Biol. Chem.* 256, 87–91] is now demonstrated in the yeast mutant GL7, which is auxotrophic for sterol and unsaturated fatty acid. Mutant cells growing poorly when provided with cholesterol and oleic acid respond to ergosterol supplements (ergosterol-to-cholesterol ratio, 1:3) by a pronounced increase in growth rates and cell yields. Stigmasterol also elicits a significant synergistic effect, and 7-dehydrocholesterol, a smaller one. Evidence for a metabolic role of ergosterol in yeast membranes is presented. Cells raised on a 1:3 mixture of ergosterol to cholesterol up to midlogarithmic phase subsequently incorporate [1-^{14}C]oleic acid at significantly faster rates into phospholipids than do cells grown on cholesterol alone.**

In a series of reports, we have described a phenomenon termed "sterol synergism" on the basis of the following observations. The sterol auxotroph *Mycoplasma capricolum* grows optimally on cholesterol but only poorly on lanosterol (1, 2). Bacterial cells supplied simultaneously with two sterols, cholesterol in limiting and lanosterol in nonlimiting quantities, grow much more rapidly and with higher yields than do cells receiving cholesterol or lanosterol alone (3). The response is synergistic, not additive. These findings were taken to indicate more than one function for sterols in membranes, and supporting evidence has been published (4). Here we describe a similar phenomenon for an eukaryotic cell, the yeast mutant GL7, chosen because it has an absolute sterol requirement for growth (5).

In designing the present experiments, we took advantage of the facts that (*i*) GL7, a mutant deficient in both squalene epoxide cyclase and heme synthesis (5), grows optimally when supplied with ergosterol and one of a variety of mono- or polyunsaturated fatty acids, but (*ii*) cholesterol, a sterol foreign to yeast, replaces ergosterol effectively only when either linoleate or a mixture of palmitoleate and oleate serves as the external fatty acid source. When oleate is the only fatty acid supplied, growth on cholesterol is much slower than on ergosterol (6). We now find that under these latter conditions, ergosterol in amounts too small to elicit detectable growth by itself raises the growth rate of GL7 cells by a large factor.

We also note that the sterol requirement for GL7 is remarkably small. Grown on a nearly optimal supply of ergosterol, these cells contain about 10% of the total sterol that wild-type yeast normally produces. Spheroplasts of GL7 cells grown on different sterol concentrations or "synergistic" sterol mixtures were fractionated, and subcellular membrane fractions were analyzed for their relative sterol and phospholipid content. Finally, we report that mutant cells raised on ergosterol/cholesterol show an enhanced capacity to incorporate external oleic acid into cellular phospholipids. This finding is in keeping with a specific role for sterols in lipid biosynthesis as first proposed and documented in the course of studies with *M. capricolum* (4).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

EXPERIMENTAL PROCEDURES

Materials and Methods. *Saccharomyces cerevisiae* strain GL7 (erg 12 heme 3) was kindly supplied by D. B. Sprinson. The mutant was grown on a synthetic minimal medium supplemented with methionine (5). Sterols (dissolved in Brij 58/ethanol, 1:4, wt/wt) were added to the medium in the concentrations indicated. Oleic acid (included in the sterol/Brij 58 solutions) was added to the medium to a final concentration of 50 μg/ml. Cultures were grown aerobically with shaking at 30°C, and growth was monitored by measuring absorbances at 540 nm. For routine experiments, starter cultures were grown on medium containing 1 μg of ergosterol per ml. Logarithmic-phase cells were centrifuged and washed twice with minimal medium, and the cell suspensions were diluted into fresh medium (1:500) containing the various sterol supplements.

Ergosterol, 7-dehydrocholesterol, cholesterol, and stigmasterol (Sigma) were recrystallized from ethanol and acetone before use. Brij 58, DNase, oligomycin, β-glucuronidase, oleic acid, concanavalin A, Tergitol NP-40, and bovine serum albumin were obtained from Sigma. Renografin was a product of Squibb. [1-^{14}C]Oleic acid was purchased from New England Nuclear.

Preparation of Spheroplasts and Plasma Membranes. Three-liter cultures of GL7 supplemented with sterol and oleic acid were grown to midlogarithmic phase and harvested. Yeast spheroplasts were obtained by treatment with snail β-glucuronidase as described by Cabib (7). Plasma membranes were separated by the method of Duran *et al.* (8), following the directions of R. Reynolds in this laboratory. Spheroplasts were suspended in 50 mM Tris buffer, pH 7.5/0.8 M sorbitol/10 mM $MgSO_4$, centrifuged, washed twice, and finally resuspended (1 ml/g of wet weight) with the same buffer throughout. For every milliliter of spheroplast suspension, 15 ml of buffer containing 0.3 mg of concanavalin A per ml was then added. After standing for 10–15 min at room temperature, spheroplasts were centrifuged at 2°C for 1 min in a desk-top clinical centrifuge. The pellet was resuspended in buffer containing 10 mM Tris, 5 mM $MgSO_4$, 0.6 mM *p*-phenylmethylsulfonyl fluoride, and DNase (8.5 mg for every 0.5 ml of original suspension). The spheroplasts were then disrupted with a Dounce homogenizer at 0°C, incubated for 15–20 min at 30°C, and stored on ice until further use. Continuous 10-ml gradients of Renografin, ranging from 5.8% (top) to 50% (bottom) mixed with 20 mM Tris (pH 7.5) were prepared for density gradient centrifugation. Spheroplast lysate (3 ml) was layered gently on top of each gradient and centrifuged in a SW 41 rotor for 1 hr at 27,000 rpm. The plasma membrane band was collected separately, whereas all of the others

* To whom reprint requests should be addressed.

were combined. The two fractions were diluted 1:10 with 5 mM Tris, pH 7.5/2 mM $MgSO_4$ and centrifuged for 30 min in a 40 rotor at 30,000 rpm.

For ascertaining the purity of plasma membranes, α-mannosidase (9), NADPH-cytochrome *c*-oxidoreductase (10), oligomycin-sensitive ATPase (11), or succinic dehydrogenase (12) were used as enzyme markers. Their absence ruled out contamination by vacuoles, microsomal membranes, and mitochondria, respectively.

Sterol and Phospholipid Concentration. Membrane lipids were extracted by the method of Bligh and Dyer (13). Samples in 1 vol of buffer were mixed with 1.25 vol of chloroform and 2.5 vol of methanol and allowed to stand for 1 hr; 1 vol of chloroform was added, and the mixture was vortexed. After the two phases had separated, the bottom layer was withdrawn for lipid analysis.

For phospholipid determination, phosphate analyses were done by the method of Chen *et al.* (14).

For sterol analysis, membranes were saponified by heating with 20% potassium hydroxide in 90% methanol at 70°C for 1 hr. Nonsaponifiable lipids were extracted with petroleum ether, dried, dissolved in chloroform, and analyzed on a Perkin–Elmer model 900 gas chromatograph with a Supelco column of 3% SP-2100 on 100/120 Supelcoport at 260°C.

Determination of Sterol and Phospholipid Content in Whole Cells. Cells harvested in logarithmic phase were washed twice with water and lyophilized. The lyophilized powder was refluxed with methanol for 1 hr, and then 2 vol of chloroform was added. The mixture was vortexed and left overnight at 4°C. The chloroform layer was collected, washed twice with 0.1 M KCl, and evaporated to dryness. The sample was then dissolved in a known volume of chloroform and divided into two portions, and sterol and phospholipid analyses were performed as described above. Sterol mol percentages were calculated from the amounts of sterol and phospholipid present.

Protein Analyses. The method of Lowry *et al.* (15) was used with bovine serum albumin as standard.

Uptake of [1-^{14}C]Oleic Acid and Incorporation into Phospholipids. GL7 cultured either in cholesterol (0.3 μg/ml) or cholesterol (0.3 μg/ml)/ergosterol (0.1 μg/ml) were grown to midlogarithmic phase (40 hr). The cells were centrifuged, washed twice with minimal medium, and added to fresh medium to a final absorbance of 0.4 at 540 nm (1.4–1.5 mg of protein/ml). The medium contained 40 μM [1-^{14}C]oleic acid (1.25 μCi/μmol; 1 Ci = 3.7×10^{10} Bq) dissolved in Brij 58/ethanol, 1:4 (wt/wt). The cell suspensions were incubated at 30°C. For determining [1-^{14}C]oleate uptake, cell samples (0.5 ml) were removed at 10-min intervals, collected on 0.45-μm Millipore filters, and then washed with 20 ml of minimal medium containing 0.5% Tergitol. For measuring radioactivity, filters were transferred to scintillation vials and dried at 100°C for 1 min, and scintillation fluid (12 ml; Scint A, United Technologies, Packard) was added. To determine fatty acid incorporation into phospholipid, cell samples (1 ml) were taken out at 10-min intervals and added to 4 ml of chloroform/methanol, 1:1 (vol/vol). The organic layer was washed with 2 ml of chloroform/methanol/water, 3:48:47 (vol/vol), applied to silica gel TLC plates (Baker), and developed in chloroform/methanol/acetone/acetic acid/water, 60:10:20:10:5 (vol/vol). The various phospholipid fractions were identified by comparison with simultaneously run authentic phospholipids. Radioactive samples were transferred to scintillation vials and assayed.

RESULTS AND DISCUSSION

We first attempted to demonstrate sterol synergism in wild-type yeast maintained anaerobically. Under these conditions, yeast has an absolute requirement for sterol (16). In such experiments, slight growth was observed with supplements of lanosterol (8 μg/ml). Ergosterol in trace quantities (0.04 μg/ml) stimulated growth several fold (M. Sobus, this laboratory, personal communication). However, the results were variable, presumably because strictly anaerobic growth conditions could not be established consistently. Nevertheless, these experiments provided the first, if tenuous, evidence that the phenomenon of sterol synergism (4, 5) is not restricted to mycoplasma.

The yeast mutant GL7, a sterol auxotroph both aerobically (5) and anaerobically (17), proved to be more suitable for our purposes. In media containing oleate as the source of unsaturated fatty acid, the minimal concentrations of single sterols that afforded nearly optimal growth after 50 hr were either 0.3 μg of ergosterol or 1 μg of cholesterol per ml, with mass doubling times of 5 and 5.5 hr, respectively (Table 1). Growth was barely detectable ($A_{540\ nm}$, <0.02) with 0.1 μg of ergosterol per ml and minimal with 0.3 μg of cholesterol per ml. However, when cultures were supplied with both 0.3 μg of cholesterol and 0.1 μg of ergosterol per ml (synergistic sterol mixture), the growth rates and final absorbances rose dramatically. A typical experiment is shown in Fig. 1. The absorbances after 50 hr were at least 5 times greater than the sum of those obtained with the two sterols individually at these concentrations. Doubling times were sharply reduced, from 12 hr for 0.3 μg of cholesterol per ml to 5 hr for cultures supplied with the synergistic sterol mixture. Similar though less-striking effects were obtained by supplementing cholesterol (0.5 μg/ml) with ergosterol (0.1 or 0.05 μg/ml) (data not shown). Thus, the yeast mutant responds to pairs of sterols in the same synergistic manner as *M. capricolum* (4). What distinguishes the two sterol auxotrophs are their sterol specificities. Yeast normally synthesizes the 24-methylcholestane derivative ergosterol. Cholesterol itself is foreign to the yeast cell. Therefore, it is the sterol that is indigenous to the species which, in subminimal amounts, promotes or stimulates yeast growth on nonlimiting quantities of a less-efficient sterol analogue. Mycoplasmas are natural sterol auxotrophs, not artificial mutants. In this instance, therefore, it is not appropriate to refer to sterols as being either natural or foreign except that, for mycoplasma strains such as *M. capricolum*, animal tissues containing cholesterol provide the natural habitat. For these cells, cholesterol may have become "natural" by adaptation. It functions in mycoplasma cells as the synergistic component, as does ergosterol in yeast.

We have shown (6) that the superior promotion of yeast growth by ergosterol is shared to a large extent by stigmasterol

Table 1. Efficiencies of ergosterol and cholesterol as growth supplements for the yeast mutant GL7

Sterol added, μg/ml	A_{540} after 50 hr	Mass doubling time, hr
Ergosterol		
10.0	1.38	3
1.0	1.20	4
0.5	0.95	4.5
0.3	0.86	5
0.1	0.02	—
Cholesterol		
10.0	1.05	5
5.0	1.03	5
1.0	0.88	5.5
0.5	0.12	8.5
0.3	0.08	12
0.1	—	—

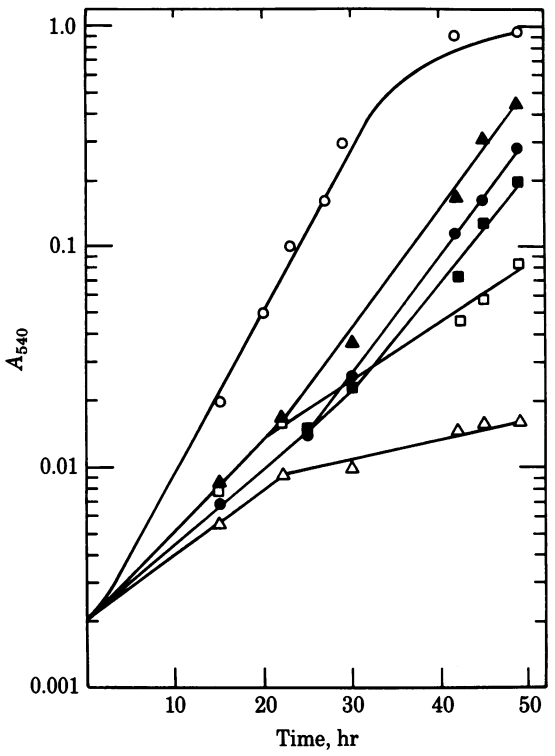

FIG. 1. Growth rates of GL7 on media supplemented with various sterols or sterol mixtures (μg/ml). △, Ergosterol (0.1), stigmasterol (0.1), or 7-dehydrocholesterol (0.1); □, cholesterol (0.3); ■, cholesterol (0.3) and 7-dehydrocholesterol (0.1); ●, cholesterol (0.3) and stigmasterol (0.1); ▲, cholesterol (0.3) and ergosterol (0.1); ○, ergosterol (0.5).

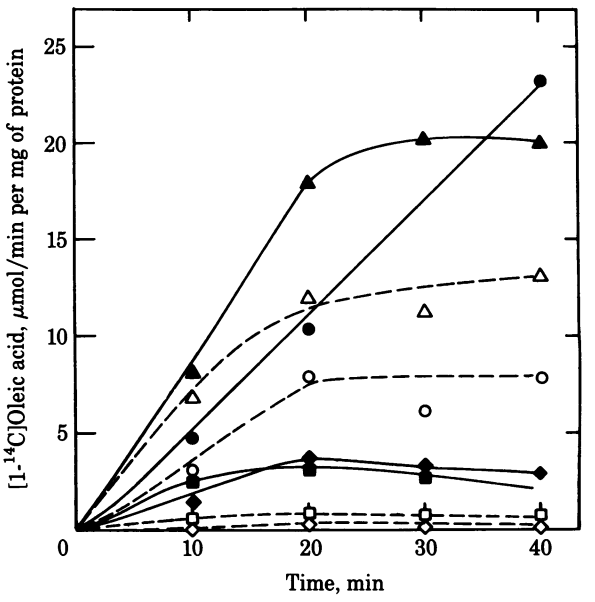

FIG. 2. Rate of oleate incorporation into phosphatidylinositol (▲, △), phosphatidylcholine (●, ○), phosphatidylethanolamine (■, □), and phosphatidylserine (◆, ◇) by cells cultured on cholesterol (0.3 μg/ml)/ergosterol (0.1 μg/ml) (——) or on cholesterol (0.3 μg/ml) alone (---).

and β-sitosterol, sterols that also have an alkylated side chain. The growth response to 7-dehydrocholesterol and the effect of this sterol on membrane phospholipid composition did not differ significantly, however, from the results obtained with cholesterol (6). Clearly, it is the alkyl group in the sterol side chain and not the 5,7-diene system in ring B that renders ergosterol functionally optimal for yeast. In the assay of GL7 cell growth for sterol synergism, the plant sterol stigmasterol was also active, though less so than ergosterol (Fig. 1). The smallest though still significant synergistic effect was shown by 7-dehydrocholesterol. From this admittedly incomplete specificity analysis, we conclude that all three structural features typical for the principal sterols of yeast and fungi—the methyl group at C_{24}, the side-chain double bond, and the 5,7-diene system of ring B—contribute to make ergosterol optimally efficient for the cells that synthesize it. (The lesser efficiency of stigmasterol can be attributed to the absence of one of these features, the Δ^7 double bond; and the relatively poor performance of 7-dehydrocholesterol can be attributed to the absence of both the Δ^{22}-double bond and the alkyl group in the side chain.)

The experiments we report here suggest that the molecular basis of the synergistic effect in the yeast mutant may be similar to that described in experiments with *M. capricolum* (4). Cells of the bacterial sterol auxotroph grown on a synergistic mixture of cholesterol and lanosterol (1:20) were found to incorporate oleate more efficiently into phosphatidylglycerol than did cells raised on lanosterol alone. In analogous experiments with GL7, cells grown on 1:3 ergosterol/cholesterol were harvested in midlogarithmic phase and then suspended in a resting medium containing [1-^{14}C]oleate. Such cells take up [1-^{14}C]oleate significantly faster than do cells grown on cholesterol alone. Large, ergosterol-stimulated increases were found in all individual phospholipids (Fig. 2). The ergosterol effects were especially evident in phosphatidylcholine and phosphatidylinositol, the two phospholipids that account for the bulk of the oleate-derived radioactivity. Under the conditions chosen, oleate incorporation into phosphatidylethanolamine and phosphatidylserine was much slower, but in these instances the response to ergosterol was equally marked. Data showing the ergosterol effect on the rate of synthesis of individual phospholipids, measured 20 min after the [1-^{14}C]oleate pulse, are given in Table 2.

Yeast, in contrast to mycoplasmas, contains more than one organellar membrane. Therefore, it will be of interest to determine whether ergosterol promotes the formation of phospholipids generally or only in selected membranes.

In the present experiments, the quantities of sterol found necessary for close to optimal growth of GL7 were more than 1 order of magnitude lower than those used in earlier studies either with anaerobic wild-type yeast (18) or with the yeast mutant GL7 (19). For example, GL7 cells raised on a limiting sterol supply (e.g., 1 μg of ergosterol per ml) contained as little as 0.04% total sterol on a dry-weight basis or about one order of magnitude less than the amounts of ergosterol produced by

Table 2. Synergistic effect of ergosterol on cellular [1-^{14}C]oleate uptake and phospholipid synthesis in the yeast mutant GL7

	A/B*
Total cells	1.33
Total phospholipid†	1.5
Phosphatidylcholine	1.5
Phosphatidylinositol	1.4
Phosphatidylserine	2.7
Phosphatidylethanolamine	3.7

* A/B represents the ratio of nmol of [1-^{14}C]oleate per mg of protein incorporated after 20 min into fractions from cells grown on cholesterol (0.3 μg/ml)/ergosterol (0.1 μg/ml) (A) to cells grown on cholesterol (0.3 μg/ml) alone (B). The ratios given represent the average values from four identical experiments.

† The total phospholipid fraction accounts for 80–85% of [1-^{14}C]oleate incorporated by the cell.

wild-type *S. cerevisiae* (20). Sterol ester formation was greatly reduced under these conditions (unpublished data). Moreover, the minimal sterol requirement of GL7 might be a consequence of the multiple enzyme deficiencies of the mutant and, hence, loss of some basic metabolic functions (e.g., respiration).

When the external ergosterol supply for GL7 was reduced from optimal (10 μg/ml) to suboptimal or marginal (0.3 μg/ml) (reduction of final absorbance from 1.38 to 0.86), the proportion of sterol in total membrane lipids decreased to about one-half of the values seen when the sterol supply was not limiting, the sterol mole percent in the total yeast lipids declining from ≈38 to 15–17% (Table 3). When membrane fractions derived from GL7 protoplasts were analyzed, the plasma membranes, adequately identified by means of marker enzymes, accounted for ≈70% of the total cellular sterol, regardless of the level of external supply. Other membrane fractions containing the remainder were not further characterized. Significantly, the sterol mole percent found in the yeast plasma membranes was also reduced to half or less when cells were supplied with limiting amounts of ergosterol or the synergistic ergosterol/cholesterol mixture. It is worth noting that in sterol-rich animal membranes, e.g., the erythrocyte and liver plasma membranes, the sterol content is ordinarily of the order of 50 mol % (21, 22). This raises the question of whether the much lower sterol concentrations (15–20 mol %) we found in GL7 plasma membranes under limiting conditions are adequate for fluidity control. This physical parameter is believed to represent the principal function of membrane-associated sterol. In fact, no evidence exists that ergosterol in yeast and cholesterol in animals function similarly.

As the present investigation shows, the phenomenon of sterol synergism is not restricted to the prokaryotic *M. capricolum* (3, 4). We conclude that in yeast as well as in mycoplasma, sterols play an additional, not previously recognized, role as membrane components. The second role is metabolic and distinguishable from the sterol-mediated control of the physical state of the membrane bilayer. For activating the metabolic, membrane-associated process, only a small fraction of the total sterol supply is needed. It also follows that the principal or single membrane sterol a given cell normally produces (i.e., the species-specific sterol or the single sterol that affords optimal growth of an auxotroph) is designed and has been selected to serve most effectively in both control processes. Broader structural specificities are allowed for the bulk sterol function that has to do with the physical state of the membranes.

The distinction we make between the two categories of sterols on the basis of function is not meant to be absolute. Whereas the "natural" or specific molecule is clearly the most effective in controlling both membrane-associated processes, the less specific or foreign sterols (cholesterol for yeast and lanosterol for mycoplasma) must and also do have some ability, albeit limited, to control the sterol-dependent metabolic events. Otherwise they would be totally ineffectual nutritionally for sterol auxotrophs. In fact, the choice of sterols with the properties described has been crucial in allowing us to detect the metabolic component of sterol function in membranes, namely the control of some step in the synthesis of unsaturated membrane phospholipids.

Table 3. Sterol composition of GL7 grown with various sterol supplements

Sterol added, μg/ml	Sterol content, mol %		
	Whole cells	Plasma membrane	All other membranes
Ergosterol, 10	38.8	37.7	32.5
Ergosterol, 1	37.4	—	—
Ergosterol, 0.4	14.8	14.3	8.3
Cholesterol, 0.3	18.0	—	—
Ergosterol, 0.1 plus cholesterol, 0.3	16.8	20.6	11.6

Rodriguez *et al.* (23) have described what we believe to be a related phenomenon but is referred to by these authors as "sparking of growth." They show that the yeast mutant FY3 fails to grow on highly purified cholestanol unless supplied with ergosterol in amounts (0.05 or 0.01 μg/ml) that by themselves do not support growth of the mutant. The question of whether "sterol synergism" and the "sparking" phenomenon are manifestations of different or identical physiological events cannot be answered in our opinion until the mode of action of trace sterols has been defined more clearly. Our earlier findings with *M. capricolum* (4) and those presented here are steps in this direction.

Other observations that are consistent with a dual role of sterols in yeast have been reported briefly by Nes *et al.* (24).

The authors are indebted to Dr. Jean S. Dahl for helpful discussions. This work was supported by grants-in-aid from the National Institutes of Health and the National Science Foundation.

1. Odriozola, J. M., Waitzkin, E., Smith, T. L. & Bloch, K. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4107–4109.
2. Dahl, C. E., Dahl, J. S. & Bloch, K. (1980) *Biochemistry* **19**, 1462–1467.
3. Dahl, J. S., Dahl, C. E. & Bloch, K. (1980) *Biochemistry* **19**, 1467–1472.
4. Dahl, J. S., Dahl, C. E. & Bloch, K. (1981) *J. Biol. Chem.* **256**, 87–91.
5. Gollub, E. G., Liu, K., Dayan, J., Adlersberg, M. & Sprinson, D. (1977) *J. Biol. Chem.* **252**, 2846–2854.
6. Buttke, T. M., Jones, S. D. & Bloch, K. (1980) *J. Bacteriol.* **144**, 124–130.
7. Cabib, E. (1971) *Methods Enzymol.* **22**, 120–122.
8. Duran, A., Bowers, B. & Cabib, E. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3952–3955.
9. Levvy, G. A. & Conchie, J. (1966) *Methods Enzymol.* **8**, 571–584.
10. Ariga, N. & Katsuki, H. (1980) *J. Biochem. (Tokyo)* **88**, 97–102.
11. Nurminen, T., Taskinen, L. & Suomalainen, H. (1976) *Biochem. J.* **154**, 751–763.
12. Merkel, G. J., Naider, F. & Becker, J. M. (1980) *Biochim. Biophys. Acta* **595**, 109–120.
13. Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917.
14. Chen, P. S., Toribara, T. Y. & Warner, H. (1956) *Anal. Chem.* **28**, 1756–1758.
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
16. Andreason, A. A. & Stier, T. J. B. (1954) *J. Cell. Comp. Physiol.* **43**, 271–281.
17. Buttke, T. M. & Bloch, K. (1980) *Biochem. Biophys. Res. Commun.* **92**, 229–236.
18. Nes, W. R., Sekula, B. C., Nes, W. D. & Adler, J. H. (1978) *J. Biol. Chem.* **253**, 6218–6225.
19. Buttke, T. M. & Bloch, K. (1981) *Biochemistry* **20**, 3267–3272.
20. Dulaney, E. L., Stapley, E. O. & Simpf, K. (1954) *Appl. Microbiol.* **2**, 371–379.
21. Nelson, G. J. (1967) *J. Lipid Res.* **8**, 374–379.
22. Dorling, P. R. & LePage, R. N. (1973) *Biochim. Biophys. Acta* **318**, 33–40.
23. Rodriguez, R. J., Taylor, F. R. & Parks, L. W. (1982) *Biochem. Biophys. Res. Commun.* **106**, 435–441.
24. Nes, W. R., Joseph, J. M. & Adler, J. H. (1981) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **40**, 1561 (abstr.).